SUPPLEMENTAL MATERIAL

Activation of Lung p53 by Nutlin-3a Prevents and Reverses Experimental Pulmonary Hypertension

Mouraret; Role of Cell Senescence in Pulmonary Hypertension

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Supplemental methods

Echocardiography

Closed-chest transthoracic echocardiography was performed in nonsedated mice as previously described[1]. Images were acquired using a 13-MHz linear-array transducer with a digital ultrasound system (Vivid 7, GE Medical Systems). Conventional measurements [LV diameters, wall thickness and thickening] were obtained serially from M-mode tracings at the level of the papillary muscles at each time point. LV end-systolic and end-diastolic volumes (LVESV and LVEDV, respectively) were calculated from the M-mode image by the "D3" respectively as LVESD3 and LVEDD3. Stroke volume (SV) was derived from the difference between LVEDV and LVESV. Cardiac output (CO) was calculated as the product of SV and heart rate[2]. The pulsed-wave Doppler recording of pulmonary flow was obtained from the parasternal short-axis view at the level of the aortic valve. The pulsed-wave Doppler sample was positioned within the pulmonary infundibulum at the tip of the pulmonary leaflets. The following variables were measured: systolic time-velocity integral of pulmonary flow (VTI, cm), pulmonary acceleration time (PAT, ms: time from pulmonary flow onset to peak velocity), ejection time (ET, ms: time from onset to end of systolic flow velocity), and the PAT/ET ratio (%)) [1]. All measures were averaged over five cardiac cycles and the reader was blinded to the treatment of the mice.

Real-time reverse-transcription quantitative PCR (RT-qPCR)

The levels of p53 downstream genes were determined by real-time reverse-transcription (RT)-qPCR using SYBR Green (Invitrogen, Cergy-Pontoise, France). To normalize for cDNA input load, mouse 18s was used as an endogenous standard. Specific primers were as follows: p21 forward, 5'-CAGATCCACAGCGATATCCA-3' and p21 reverse, 5'-

ACGGGACCGAAGAGACAAC-3'; Bax forward, 5'-AGGATGCGTCCACCAAGAAGCT-3' and Bax reverse, 5'- TCCGTGTCCACGTCAGCAATCA-3'; Bcl2 forward, 5'-GGGAGGATTGTGGCCTTCTT-3' and Bcl2 reverse, 5'-CATCCCAGCCTCCGTTATCCT-3'; Puma forward, 5'- ACCGCTCCACCTGCCGTCAC-3' and Puma reverse, 5'-ACGGGCGACTCTAAGTGCTGC-3'; BTG2 forward, 5'-GAGCGAGCAGAGACTCAAGGTT-3' and BTG2 reverse, 5'-CGATAGCCAGAACCTTTGGATGG-3'. After addition of cDNA to SYBR Green Master Mix PCR (300 nM of each specific primer, PCR was performed in a total volume of 25 µL in a 7900HT real-time PCR cycler (Applied Biosystems, Courtaboeuf, France). All cDNA samples were tested in duplicate and analyzed using ABI Prism Sequence Detection Software v.1.7 (PE Applied Biosystems). Samples were compared using the relative Ct method. The Ct value, which is inversely proportional to the initial template copy number, is the calculated number of cycles with a fluorescence signal significantly above background levels. Fold induction or repression was measured relative to controls and calculated after adjusting for 18s using $2^{-[\Delta\Delta Ct]}$, where ΔCt = Ct tested gene – Ct 18s and $\Delta\Delta$Ct = ΔCt control – ΔCt treatment.

Western Blotting

Tissues were sonicated in lysis buffer (Cell Signaling Technology, Beverly, MA). The lysate was centrifuged for 10 minutes at 2000 rpm at 4°C and the supernatant was kept frozen. The protein concentration of each sample was determined using the Bradford method. Samples were subjected to electrophoresis in 10% or 15% polyacrylamide gels under reducing conditions. After electrophoresis, proteins from the gel were electroblotted onto polyvinylidene difluoride membranes (Millipore, Molsheim, France) for 2 h. After transfer, the membranes were saturated with phosphate buffer saline (PBS)/5% milk then probed

overnight at 4°C with antibodies. The antibodies used were anti-P-p53 Ser15 (Cell Signaling Technology, MA, USA) at a final dilution of 1:1000 ; monoclonal anti p53 (Cell Signaling Technology) at a final dilution of 1:1000; monoclonal anti-p21Waf1/Cip1 antibody (Cell Signaling Technology) at a final dilution of 1:1000, or polyclonal anti-MDM2 (Abcam, Cambridge, UK) at a final dilution of 1:1000. After three 5-minute washes in TBS-T, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies at a final dilution of 1:1000 and washed twice for 5 minutes in TBS-T. Proteins were visualized by electrochemiluminescence (Amersham, Little Chalfont, UK) according to the manufacturer's instructions. Membranes were re-probed with anti-β-actin or anti-GAPDH antibody (Sigma, Saint-Quentin-Fallavier, France) at a final dilution of 1:1000 for normalization. Complementary experiments were performed in mouse tissue to assess phosphorylated-p53 levels by using a co-immunoprecipitation procedure or to determine the nuclear and cytoplasmic fractions of total p53 protein.

Immunoprecipitation

Immunoprecipitation was carried out at 4°C. Lung tissue was sonicated in lysis buffer (Cell Signaling Technology, MA, USA). The lysate was centrifuged for 10 minutes at 2000 rpm at 4°C and the supernatant was kept frozen. After assessment of the protein concentration, 1 mg of protein from each sample was incubated with the p53 (1C12) antibody (Cell SignalingTechnology) overnight then with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h. The protein–antibody complexes recovered on beads were subjected to Western blot analysis after separation by SDS–PAGE. The membrane was probed with phosphorylated-p53 (Ser15) primary antibody (Cell Signaling Technology) and with a horseradish-peroxidase-conjugated secondary antibody. Blots were visualized by electrochemiluminescence.

Cytosolic and nuclear fraction isolation

To obtain the nuclear fraction, lung tissue was incubated in hypotonic buffer (20 mM Tris HCl, pH 7.4; 10 mM NaCl; 3 mM MgCl2) for 15 min at 4°C. Triton (0.3%) was added to the lysate, which was then centrifuged at 14,000 *g* for 10 min at 4°C and separated into soluble and insoluble fractions. The insoluble fraction containing the cytosolic protein was kept at -80°C, and the pellet containing the nuclei was incubated in lysis buffer (Invitrogen, Carlsbad, CA, USA) for 30 min. After extraction, the sample was centrifuged at 14,000 g for 10 min at 4°C, and the supernatant constituted the nuclear fraction. Cytosolic and nuclear protein samples were analyzed by Western blotting. The membranes were incubated with the appropriate primary antibody, p53 (1C12) antibody (Cell Signaling Technology), lamin A/C antibody (Santa Cruz), or alpha-tubulin (Cell Signaling Technology).

Transient tranfection of pulmonary-artery smooth muscle cells

Cells were seeded in 24-well plates and transiently transfected the following day with a p53 reporter construct (p53 Cignal Reporter Assay, Qiagen, Courtaboeuf, France), as well as with positive and negative controls, using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. After 18 h of transfection, the medium was changed and replaced by complete growth medium, and the cells were treated with Nutlin-3a 2.5 µM, 5 µM, 10 µM, or ethanol alone. A dual-luciferase assay (Promega, Charbonnières les Bains, France) was performed 24 h after treatment. Cells were lysed with lysis buffer, and luciferase activity was quantified using a Tristar luminometer (Berthold, Thoiry, France). Values are expressed as arbitrary units using a construct constitutively expressing Renilla luciferase for internal normalization. Firefly/Renilla activity ratios were generated from experimental and control transfections. Experiments were done in triplicate.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 20 minutes. Tissue was permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% methanol in PBS for 10 minutes. Slides were incubated for 60 minutes in 1% bovine serum albumin and 5% goat serum in PBS then incubated overnight with anti-p21 mouse antibody (1:200, Santa Cruz Biotechnology) and anti-Ki67 rabbit antibody (1:300, Abcam). We used the ABC Vectastain kit (Vector Labs, Burlingame, CA, USA) to mark the primary antibodies according to the user's guide. The staining substrate was diaminobenzidine (FastDAB, Sigma-Aldrich, St Louis, MO, USA) and the sections were counterstained with methyl green.

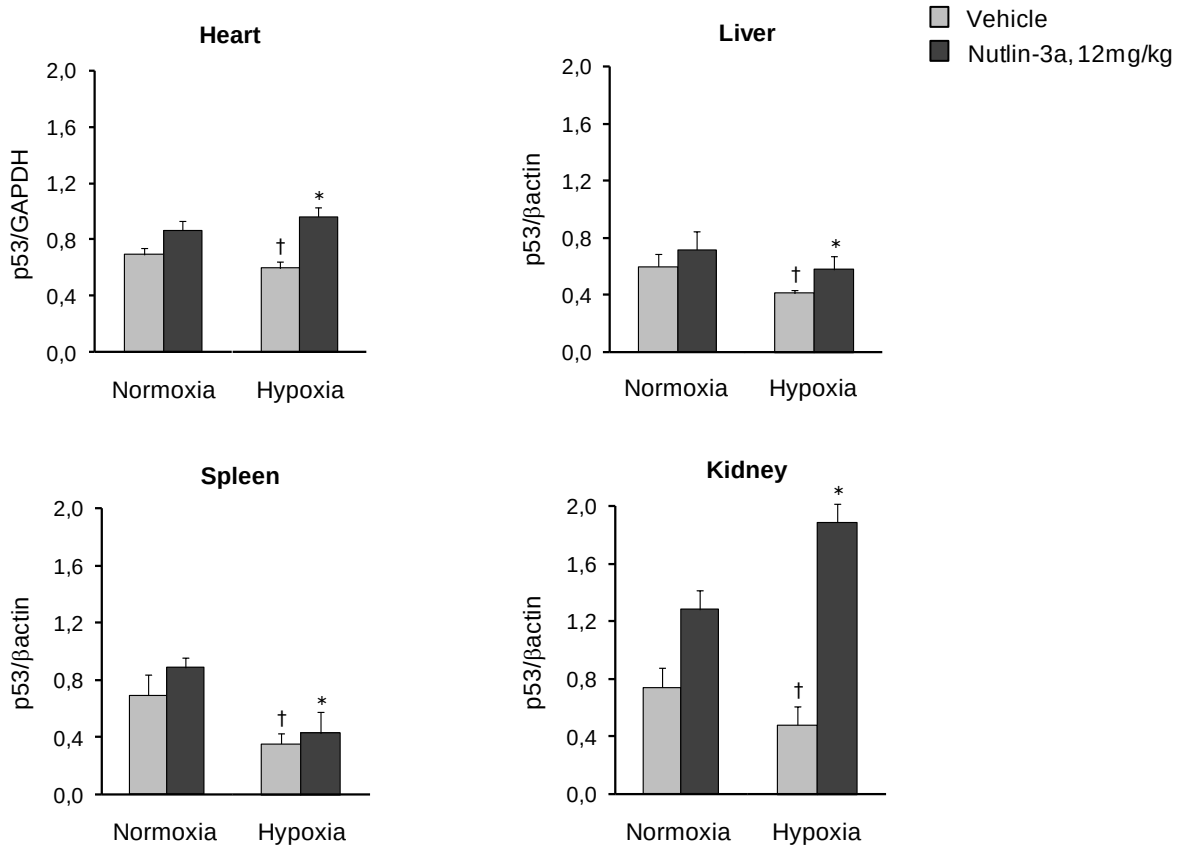
For terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), epitopes were retrieved by treatment with DNase1 digestion for 10 min at room temperature. After endogenous peroxidase blockade with H_2O_2, tissues were incubated in equilibration buffer and treated with terminal deoxynucleotidyl transferase (TdT) to detect TUNEL-positive nuclei as suggested by the manufacturer (Roche, Meylan, France). Tissues were then incubated with peroxidase-conjugated anti-digoxigenin antibodies and color was developed with DAB. After counterstaining with methyl green, sections were protected with coverslips secured with mounting medium. For immunofluorescence, slides were incubated overnight at 4°C with anti-MDM2 SMP-14 mouse antibody (1:50, Santa Cruz Biotechnology) and anti-αSMA rabbit antibody (1:200, Abcam) then exposed to anti-mouse Alexa Fluor (1:1000, Cell Signaling technology) and anti-rabbit Alexa Fluor (1:1000, Invitrogen) antibodies. Nuclei were stained with Hoechst.

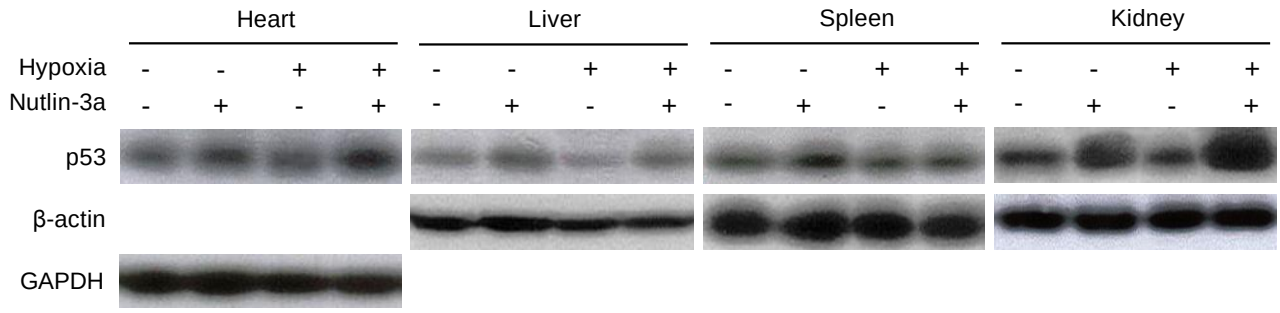
Online Table I

	Normoxia Vehicle	Normoxia Nutlin-3a	Hypoxia Vehicle	Hypoxia Nutlin-3a	*P* value
HR, bpm	658±4	665±13	620±9	653±5 *****	0.03
LVEF, %	87±4	83±2	83±3	82±2	0.57
CO, mL/min	33.4±1.8	35.8±2.4	36.8±3.5	33.7±1.9	0.64
Pulmonary TVI, cm	3.3±0.1	3.2±0.1	2.5±0.1†	2.3±0.1	0.001
PAT, ms	19.6±0.7	20.8±0.9	11.8±0.6†	14.1±0.8 *****	0.001
ET, ms	52.8±1.9	51.8±0.8	49.4±1.3	47.0±0.8	0.02
PAT/ET, %	0.37±0.02	0.40±0.02	0.24±0.02†	0.30±0.02 *****	0.001

Echocardiographic parameters in nonsedated mice. Data are mean±SEM. **P*<0.05 for comparison between hypoxic mice treated with Nutlin-3a and control untreated hypoxic mice. † p<0.05 for comparison between vehicle-treated hypoxic and normoxic mice. HR, heart rate; LVEF, left ventricular ejection fraction; CO, cardiac output; TVI, systolic time-velocity integral of pulmonary flow; PAT, pulmonary acceleration time; ET, ejection time.

Online Figure 1





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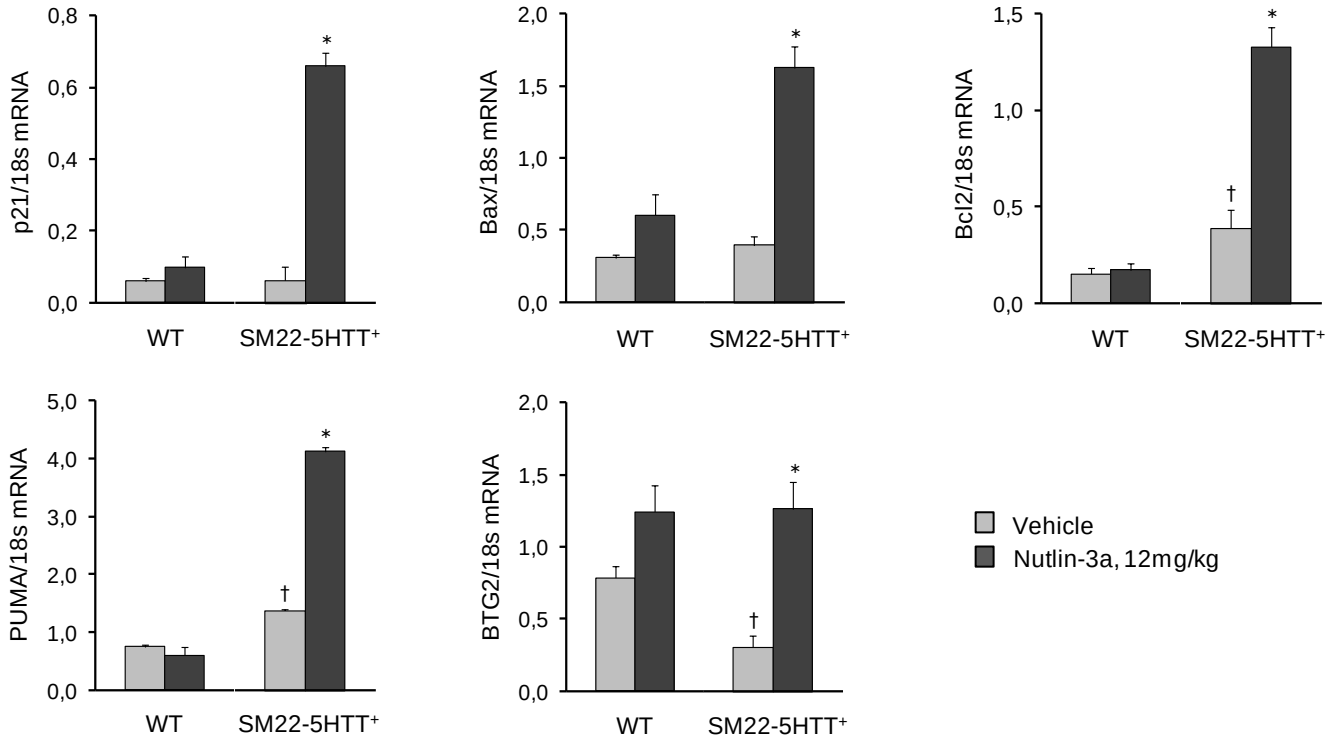

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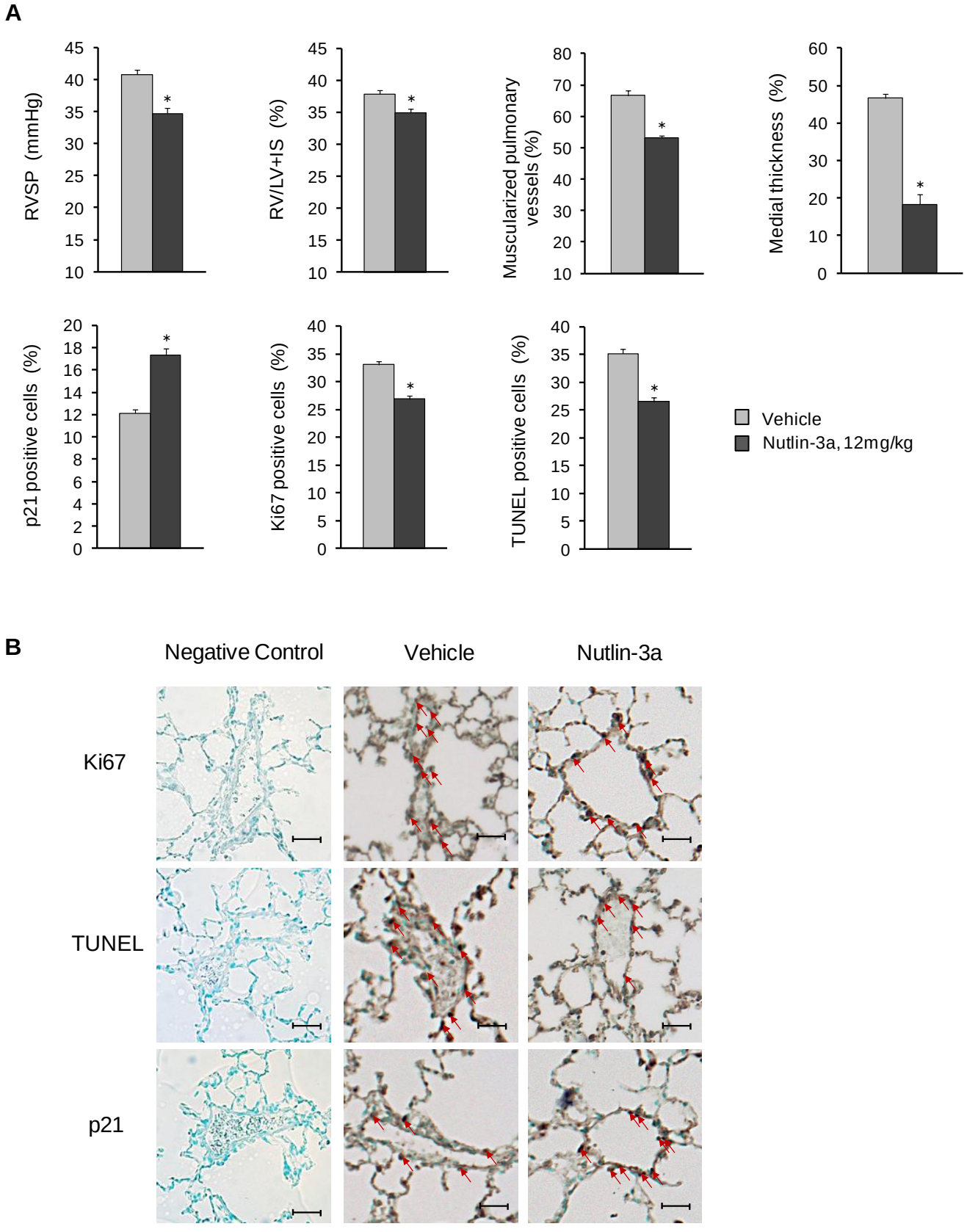

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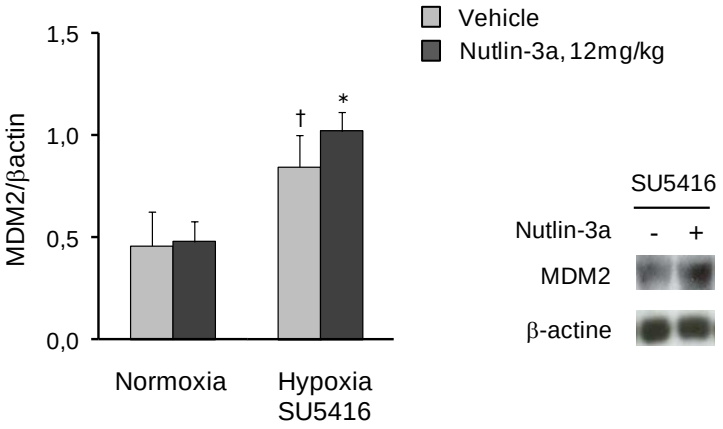

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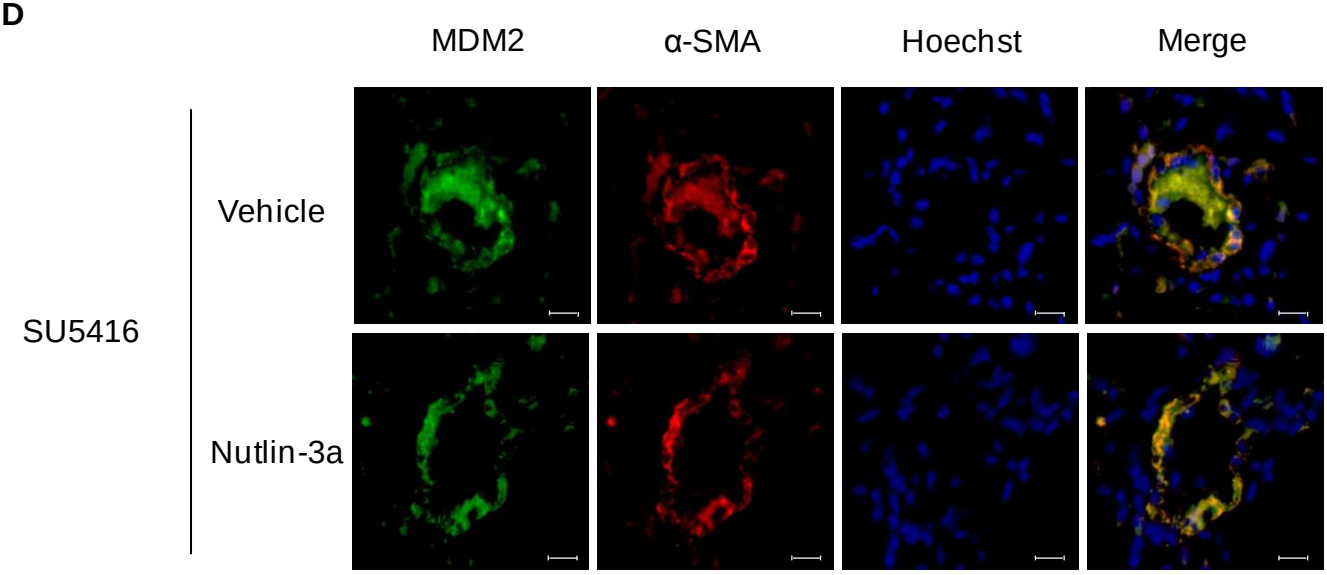

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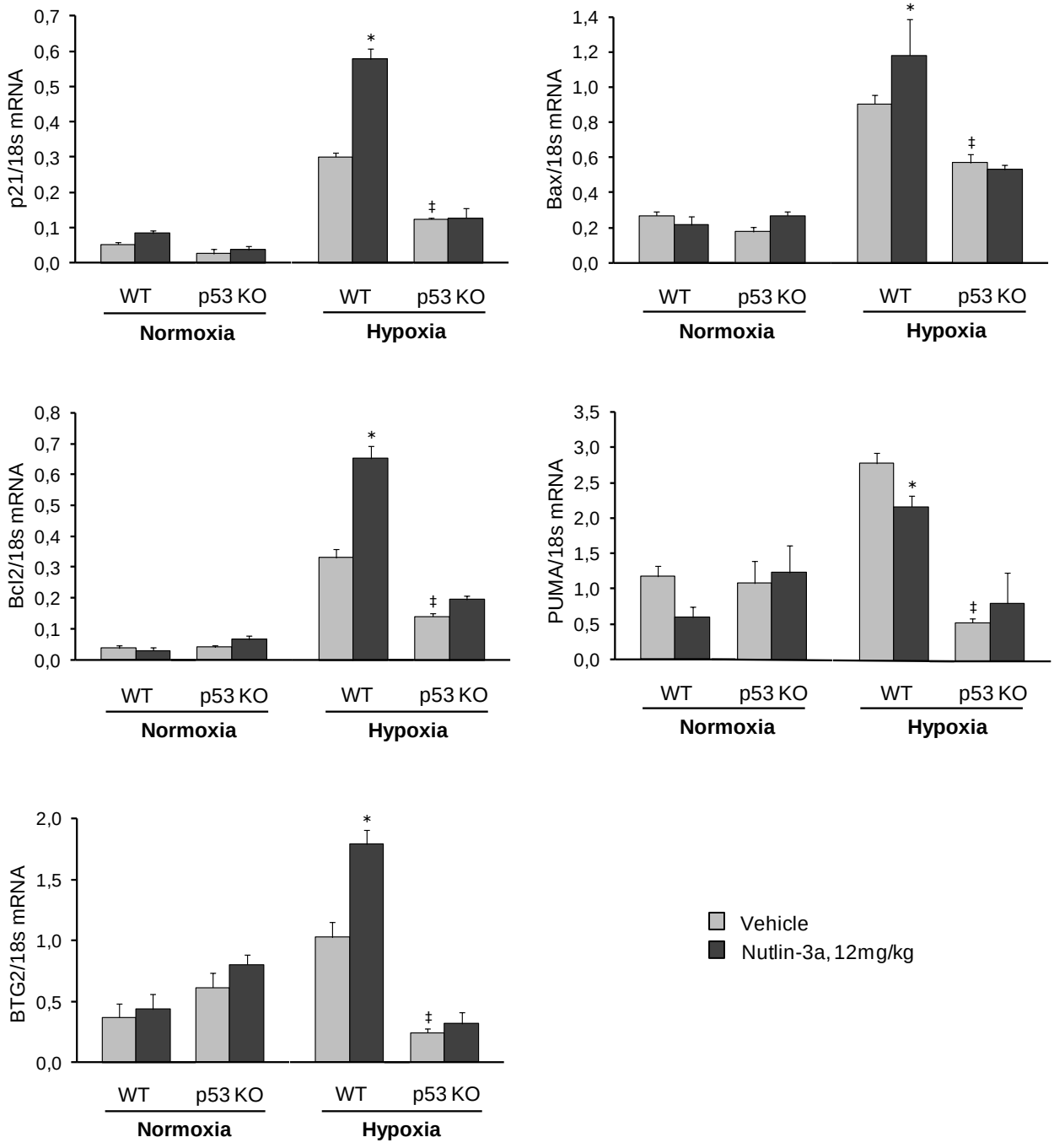
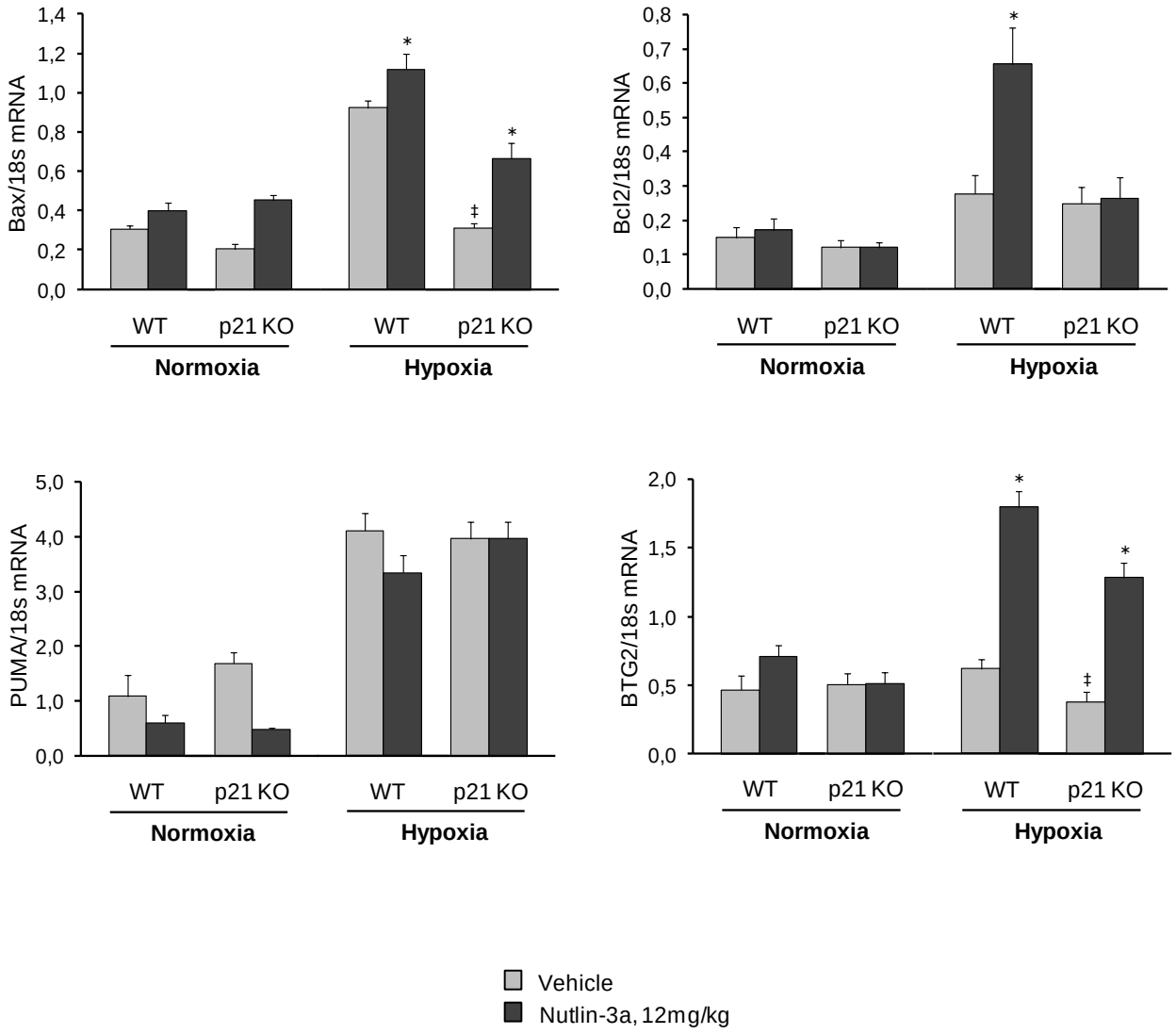


Online Figure 5



Online figure legends

Online Figure 1

Protein p53 levels measured by Western blot in the heart, liver, spleen, and kidneys in mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 25 mg/Kg Nutlin-3a. Mice were sacrificed exactly 3 hours after the last Nutlin-3a dose. **P*<0.05 compared with values in vehicle-treated mice after exposure to hypoxia or normoxia. Data are mean±SEM of 6 to 10 animals. †*P*<0.05 compared with values in vehicle-treated mice after exposure to hypoxia.

Online Figure 2

Lung mRNA levels of p21, bax, Bcl2, BTG2, and PUMA measured by RT-PCR in SM22-5-HTT+ mice and control mice studied after 21 days of treatment with vehicle or 12 mg/Kg of Nutlin-3a. Mice were sacrificed exactly 3 hours after the last Nutlin-3a dose. Data are mean±SEM of 6 to 10 animals. **P*<0.05 compared with values in vehicle-treated mice. †*P*<0.05 for SM22-5HTT+ versus vehicle-treated control mice.

Online Figure 3

A, right ventricular systolic pressure (RVSP); right ventricular hypertrophy index (RV/[LV+S] weight ratio); pulmonary vessel muscularization (percentage of partially and fully muscularized pulmonary vessels); medial wall thickness of fully muscularized intraacinar arteries; and percentages of Ki67-positive dividing cells, p21-stained cells, and TUNEL-positive cells in mice studied on day 21 after exposure to hypoxia and simultaneously given a weekly intraperitoneal injection of 20 mg/Kg SU5416, and treated daily by i.p. injections of vehicle or Nutlin-3a. **B**, representative micrographs of pulmonary

vessels stained for Ki67, TUNEL, or p21. No immunoreactivity was detected in sections incubated with secondary anti-rabbit and anti-mouse antibody but no primary antibody (Negative controls). **C**, Lung protein levels of MDM2 measured by Western blot. **D**, representative photographs of immunofluorescence staining for MDM2 in mice subjected to SUGEN/hypoxia and treated with Nutlin-3a or vehicle for 21 days. MDM2 was predominantly expressed in pulmonary vessels and co-localized with α-SMA in smooth muscle cells (SMCs). Data are mean±SEM of 6 to 10 animals.*P<0.05 compared with values in vehicle-treated mice.

Online Figure 4

Lung mRNA levels of p21, Bax, Bcl2, PUMA, and BTG2 in wild-type and p53 knockout (KO) mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 12 mg/Kg Nutlin-3a. Data are mean±SEM of 10 animals. *P<0.05 compared with values in vehicle-treated mice. ‡P<0.05 compared with values in vehicle-treated control mice.

Online Figure 5

Lung mRNA levels of Bax, Bcl2, PUMA, and BTG2 in wild-type and p21 knockout (KO) mice studied on day 21 after exposure to hypoxia or normoxia and treated with daily i.p. injections of 12 mg/Kg Nutlin-3a. Data are mean±SEM of 6 to 10 animals. *P<0.05 compared with values in vehicle-treated mice. ‡P<0.05 compared with values in vehicle-treated control mice.

References

1. Thibault HB, Kurtz B, Raher MJ, Shaik RS, Waxman A, Derumeaux G, Halpern EF, Bloch KD, Scherrer-Crosbie M. Noninvasive assessment of murine pulmonary arterial pressure: validation and application to models of pulmonary hypertension. *Circ Cardiovasc Imaging.*3(2):157-163.

2. Tournoux F, Petersen B, Thibault H, Zou L, Raher MJ, Kurtz B, Halpern EF, Chaput M, Chao W, Picard MH, Scherrer-Crosbie M. Validation of noninvasive measurements of cardiac output in mice using echocardiography. *J Am Soc Echocardiogr.*24(4):465-470.